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Expression of Solvent-Forming Enzymes and Onset of Solvent Production in Batch Cultures of *Clostridium beijerinckii* ("*Clostridium butylicum*")

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***Clostridium beijerinckii* ("*Clostridium butylicum*") NRRL B592 and NRRL B593 were grown in batch cultures without pH control. The use of more sensitive and accurate procedures for the determination of solvents in cultures led to the recognition of the onset of solvent production about 2 h earlier than the previously assigned point and at a higher culture pH for both strains. Reliable assays for solvent-forming enzyme activities in cell extracts have also been developed. The results showed that activities of solvent-forming enzymes in strain NRRL B592 started to increase about 1 h before the measured onset of solvent production and that the increase in activities of solvent-forming enzymes was not simultaneous. The degree of increase of these enzyme activities for both strains ranged from 2- to 165-fold, with acetoacetate decarboxylase and butanol-isopropanol dehydrogenase showing the largest activity increases. However, the pattern of increase of enzyme activities differed significantly in the two strains of *C. beijerinckii*. When an increase in solvent-forming enzyme activities was first detected in strain NRRL B592, the culture pH was at 5.7 and the concentrations of total acetic and butyric acids were 5.2 and 3.6 mM, respectively. For strain NRRL B593, the corresponding pH was 5.5. Thus, the culture conditions immediately preceding the expression of solvent-forming enzyme activities differed significantly from those that have been correlated with the production of solvents at later stages of growth.**

Acetone, *n*-butanol, ethanol, and isopropanol (solvents) are characteristic products of several *Clostridium* species (6, 7, 13, 14). While *Clostridium acetobutylicum* and some strains of *Clostridium beijerinckii* ("*Clostridium butylicum*") produce acetone, butanol, and ethanol, certain strains of *C. beijerinckii* characteristically produce isopropanol, butanol, and ethanol as the final products (6). Alcohols produced by these fermentations are useful both as chemical feedstocks and as fuel additives.

Solvent production is a regulated metabolic process. In a normal batch culture, solvent-producing *Clostridium* species produce acetate and butyrate during the exponential growth phase. Only during the late growth phase does the metabolism shift to rapid solvent production. This was first shown by Peterson and Fred (20) and Davies and Stephenson (9) and also by more recent studies with *C. acetobutylicum* (see e.g., references 1, 2, 11, 18, and 22). The metabolic shift is also observed in *C. beijerinckii* (6, 12). Therefore, solvent fermentation is generally divided into two phases: the acid-producing or acidogenic phase and the solvent-producing or solventogenic phase. It is expected that the shift in metabolic activity, which occurs when cells switch from the acid-producing phase to the solvent-producing phase, is accompanied by a corresponding shift in the cellular content of enzymes involved in the acid- and solvent-producing pathways (Fig. 1). The enzymes specifically involved in solvent production are expected to be synthesized or activated or both before the shift to solvent production can occur. The external signals that trigger the metabolic shift have not been determined (for a recent review, see reference 19).

The onset of the solventogenic phase is empirically assigned, and the accuracy of the assignment is limited by the sensitivity of assays that detect the first sign of solvent accumulation. During the period when a culture is shifting to solvent production, the culture conditions undergo rapid changes. Therefore, the assigned time of onset significantly affects the culture conditions to be considered as possible triggering signals. It is possible that the threshold external conditions which trigger the metabolic switch differ from the more readily identified culture conditions (in batch or continuous cultures) that are conducive to sustained solvent production. Knowledge of the more precise moment of the beginning of the metabolic transition allows the identification of more pertinent external conditions and physiological states as possible factors involved in the triggering of the metabolic transition. For this purpose, activities of solvent-forming enzymes can serve as early indicators of the onset of the solventogenic switch. It is also important to determine whether these enzyme activities appear (or increase) in coordination or independently, since this information could bear on the elucidation of the control mechanism for the solventogenic switch and could affect approaches to be taken to improve the fermentation.

Changes in acid- and solvent-forming enzyme activities during growth of *C. acetobutylicum* have been reported (2, 10, 15, 22), but different patterns of activity changes were observed between laboratories. During this study, we first controlled the growth stage of the inocula and then measured the cellular levels of enzymes responsible for acetone, butanol, and isopropanol formation (reactions 1 to 5 in Fig. 1), in *C. beijerinckii* throughout the entire growth period. (The presence of these solvent-forming enzymes in *C. beijerinckii* has been reported previously [12, 17; R.-T. Yan and J.-S. Chen, Fed. Proc. **46**:1951, 1987; S. F. Hiu, W.-L. Fan, W. R. Aimutis, and J.-S. Chen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, O83, p. 249].) In addition, we measured the cellular level of thiolase and glucose-6-phosphate isomerase for comparison. Cell density, culture pH, and the level of fermentation products in the growth medium were monitored at frequent intervals to determine the culture

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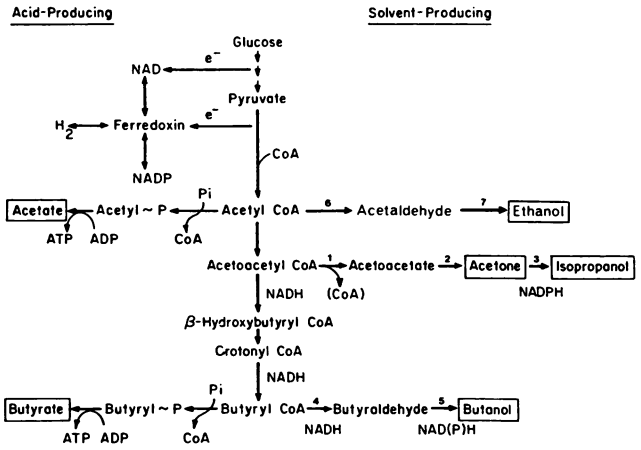

FIG. 1. Metabolic pathway of acetone-butanol-isopropanol-ethanol fermentation. Key enzymes involved in the final reactions of solvent production are (reaction 1) acetoacetyl-CoA:acetate/butyrate-CoA transferase or acetoacetyl-CoA hydrolase or both, (reaction 2) acetoacetate decarboxylase, (reaction 3) isopropanol dehydrogenase, (reaction 4) butyraldehyde dehydrogenase, (reaction 5) butanol dehydrogenase, (reaction 6) acetaldehyde dehydrogenase, and (reaction 7) ethanol dehydrogenase.

conditions at the onset of solvent production. (A preliminary report of this work was presented at the American Society for Microbiology annual meeting on 1 to 6 March, 1987, in Atlanta, Ga. [Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, O-58, p. 270].)

MATERIALS AND METHODS

Materials. Tryptone and yeast extract were obtained from Difco Laboratories, Detroit, Mich.; dithiothreitol, DNase I (DN-25), NAD, NADP, NADPH, glycylglycine, coenzyme A (CoA), glucose-6-phosphate dehydrogenase (from bakers' yeast), fructose 6-phosphate (disodium salt), acetoacetyl-CoA, acetoacetic acid (lithium salt), *n*-butyric acid, biotin, and Trizma base were obtained from Sigma Chemical Co., St. Louis, Mo.; the dye-binding protein assay kit was obtained from Bio-Rad Laboratories, Richmond, Calif.; α-toluenesulfonyl fluoride was obtained from Eastman Kodak Co.; *n*-butanol, isopropanol, acetone, butyraldehyde, and glacial acetic acid were obtained from Fisher Scientific Co., Pittsburgh, Pa.; methanol was obtained from Burdick and Jackson Laboratories Inc., Muskegon, Mich.

Organisms and growth conditions. *C. beijerinckii* NRRL B592 (VPI 13436; the strain not producing isopropanol) and NRRL B593 (VPI 13437; the strain producing isopropanol) were used in this study. For each growth experiment, 6 ml of a spore suspension was heated in boiling water for 1 min, transferred to 50 ml of a chopped-meat–carbohydrate (CMC; meat removed) medium (12) under N_2, and incubated at 35°C with shaking for 13 to 18 h. The culture, which was at the exponential growth stage, was transferred to 1 liter of a tryptone-yeast extract-sucrose (TYS) medium (12), which was then incubated at 30°C with stirring for 3.75 to 5.75 h. Finally, the 1-liter culture (in a 1-liter Erlenmeyer flask fitted with gassing and sampling ports), which was at the exponential growth stage, was used to inoculate 7 liters of TYS medium, and this 8-liter culture (in a 9-liter glass serum bottle fitted with gassing and sampling ports) was incubated at 30°C with stirring and monitored for 29 to 30 h (entire growth period) or 6 h (early phase). During growth, frequent samples were taken for the measurement of cell density, culture pH, and product concentrations, and up to seven samples were taken for the measurement of enzyme levels. The 1- and 8-liter cultures were initially kept under N_2 and were then vented through a bubbler during growth to maintain a 1-atm (101.3-kPa) gas phase. During sampling, N_2 was used to maintain the gas phase slightly above 1 atm to displace a desired volume of culture through glass tubing. Cells were sedimented by centrifugation, and culture supernatants were stored at −20°C until analyzed. For enzyme analysis, sedimented cells were washed once in 50 mM Tris chloride buffer (pH 8) and stored at −80°C for up to 4 days before cell extracts were prepared. Growth was monitored at 550 nm with a Hitachi 100-40 spectrophotometer.

Preparation of cell extracts. Cell paste was thawed under argon in 50 mM Tris chloride (pH 8) (3 ml/g of cell paste) containing DNase I (0.1 mg/ml) and α-toluenesulfonyl fluoride (0.3 mg/ml) as a protease inhibitor. All operations were carried out under argon. Cells were disrupted by two passages through a French pressure cell at 18,000 lb/in^2, and suspensions of broken cells were incubated at room temperature for 15 min to allow DNase I to decrease the viscosity. Cell debris were then removed by centrifugation at 37,000 × *g* for 30 min at 4°C. The supernatant (cell extract) was stored as frozen pellets in liquid nitrogen.

Analytical procedures. Solvents and acids in culture supernatants were measured with a Gow-Mac series 750 FID gas chromatograph (Gow-Mac Instrument Co., Bridgewater, N.J.) linked to an HP 3390A integrator (Hewlett Packard Co., Avondale, Pa.). Quantification of acids in culture supernatants by gas chromatography was prone to large variations. Therefore, each sample for acid determination was measured up to 20 times (10 times on the average), so that the coefficients of variation (standard deviation divided by mean) were below 0.15.

Solvent concentrations were determined by using a glass column of 2 m by 4 mm containing 80/100 mesh Carbopack C/0.1% SP-1000 (Supelco, Inc., Bellefonte, Pa.). The column temperature was 80°C; the injector and detector temperature was 150°C. The flow rate of the carrier gas, nitrogen, was 30 ml/min. For samples containing low concentrations of solvents (less than 1 mM butanol), 5 μl of the sample was used, and for samples with higher concentrations of solvents, 1 μl of the sample was used. In more recent experiments, better resolution was obtained by injecting 1 μl of sample onto a glass column of 2 m by 2 mm containing the same packing, with the column temperature at 75°C and the injector and detector temperature at 150°C.

Acid concentrations were determined on a glass column (2 m by 2 mm) containing Chromosorb 101 (Supelco, Inc., Bellefonte, Pa.). The column temperature was 165°C; the injector and detector temperature was 210°C. The flow rate of the carrier gas (nitrogen) was 25 ml/min. We used 1 μl of sample. The samples were acidified first by adding 20 μl of 1 N HCl to 100 μl of each sample before injection.

Protein was determined by the dye-binding assay (5) with bovine serum albumin as a standard.

Enzyme assays. NAD(P)H-linked enzymes in crude extracts were assayed under anaerobic conditions to circumvent the interference of diaphorase activities (17). Other enzymes were stable in air and were assayed under aerobic conditions. The volume of reaction mixture for spectrophotometric assays was 1 ml. Alcohol dehydrogenase activities were determined in the direction of butyraldehyde or acetone reduction (17). *n*-Butyraldehyde dissolves slowly in water, and a clear aqueous solution is not readily formed. To

solve this problem, we dissolved butyraldehyde in methanol before use (17). Butyraldehyde dehydrogenase activity was measured by an assay modified from that of Rogers (22). The assay mixture contained 50 mM glycylglycine buffer (adjusted to pH 9.0 with NaOH), 1 mM dithiothreitol, 0.5 mM coenzyme A, 3 mM NAD^+, and 10 to 40 μl of cell extracts. The reaction was initiated by the addition of butyraldehyde (11 mM; butyraldehyde was first diluted 10-fold with methanol).

The enzyme(s) which catalyzes acetoacetate formation from acetoacetyl-CoA was measured by monitoring the disappearance of acetoacetyl-CoA. The assay mixture contained 175 mM Tris chloride buffer (pH 7.5), 20 mM $MgCl_2$, 0.1 mM acetoacetyl-CoA, 100 mM potassium acetate or butyrate, and 20 μl of cell extracts. Potassium acetate or butyrate was added last during the assay. Two activities (acetoacetyl CoA:acetate/butyrate CoA transferase and acetoacetyl-CoA hydrolase) have been found in *C. beijerinckii*, and the hydrolase activity was potassium activated (Yan and Chen, Fed. Proc., 1987). Since acetate, butyrate, and potassium are present in cell extracts, the activities (after corrections for nonenzymatic hydrolysis of acetoacetyl-CoA) observed before the addition of potassium acetate or butyrate could be attributed to acetoacetyl-CoA:acetate/butyrate CoA-transferase and acetoacetyl-CoA hydrolase activities from endogenous substrates and were thus not subtracted from the final activities. Acetoacetate decarboxylase activity was measured by manometry at 30°C as described by Davies (8), and 5 to 300 μl of cell extracts was used. Thiolase activity was assayed in the direction of acetyl-CoA formation from acetoacetyl-CoA and CoA. The assay mixture contained 105 mM Tris chloride buffer (pH 7.5), 24 mM $MgCl_2$, 90 μM acetoacetyl-CoA, and 1 μl of cell extracts. The reaction was initiated by addition of 60 μM CoA. Glucose-6-phosphate isomerase activity was assayed as described by Bergmeyer (4), except that 50 mM Tris chloride buffer (pH 7.5) was used.

RESULTS

When solvent-producing clostridia are grown in batch cultures, it is possible that the cellular level of solvent-forming enzymes is subjected to different types of controls: the level could increase when cells switch from acid production to solvent production, or the level could decrease or remain unchanged when cells at the solvent-producing stage are transferred into fresh growth medium. To minimize the last complications, only cultures at the early exponential growth phase, which showed no solvent production, were used as inocula during this study. To locate the very earliest moment of the solventogenic switch, the assays used in this study for enzyme activities and fermentation products were adapted for sensitivity and reproducibility. Whenever possible, enzyme activities were measured in the physiological direction. Changes in the activity levels of enzymes involved in acetone-isopropanol and butanol formation (Fig. 1, reactions 1 to 5) during the growth of *C. beijerinckii* NRRL B592 and NRRL B593 are shown in Fig. 2 and 3. To resolve the order of the appearance of (or increases in) these enzyme activities and to deduce the limiting enzyme(s) for the start of solvent production, we performed an experiment in which seven samples for enzyme analysis were taken during the transition period (Fig. 4).

The improved assay procedures permitted reliable measurements of solvents at concentrations below 0.1 mM, which led to the recognition of the beginning of solvent

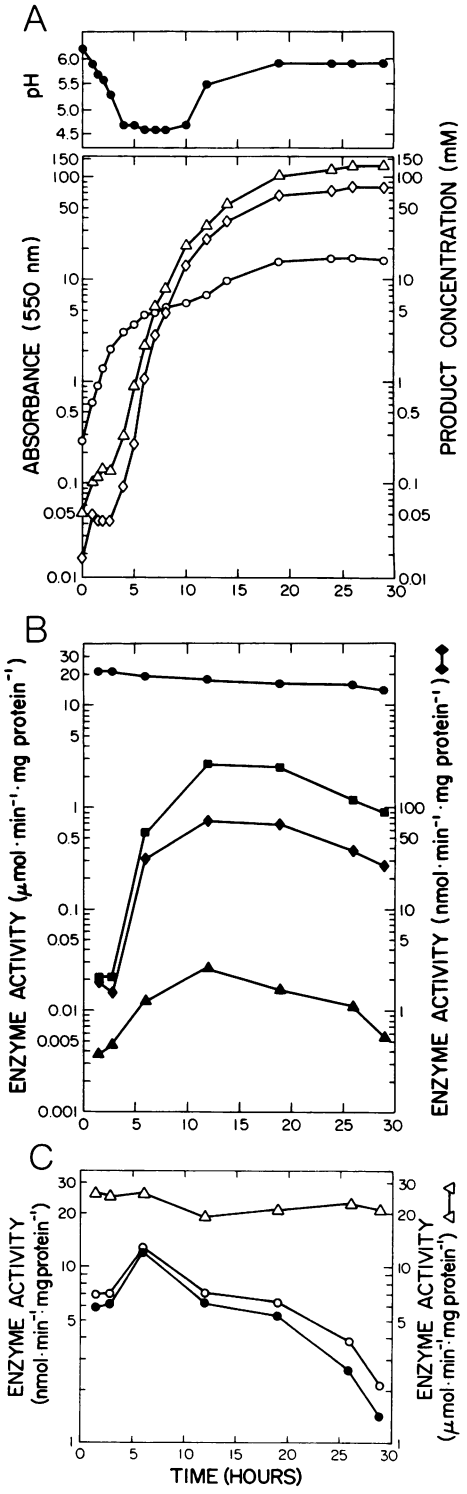

FIG. 2. Growth, culture pH, solvent production, and specific activity of solvent-forming enzymes of *C. beijerinckii* NRRL B592 in TYS medium. (A) A_{550} (○), acetone (◇), butanol (△), and pH (●). (B) Acetoacetate decarboxylase (■), butyraldehyde dehydrogenase (◆), butanol dehydrogenase (▲), and glucose-6-phosphate isomerase (●). (C) Acetoacetate-forming (acetoacetyl-CoA–utilizing) enzymes in the presence of potassium acetate (○) or potassium butyrate (●), and thiolase (△).

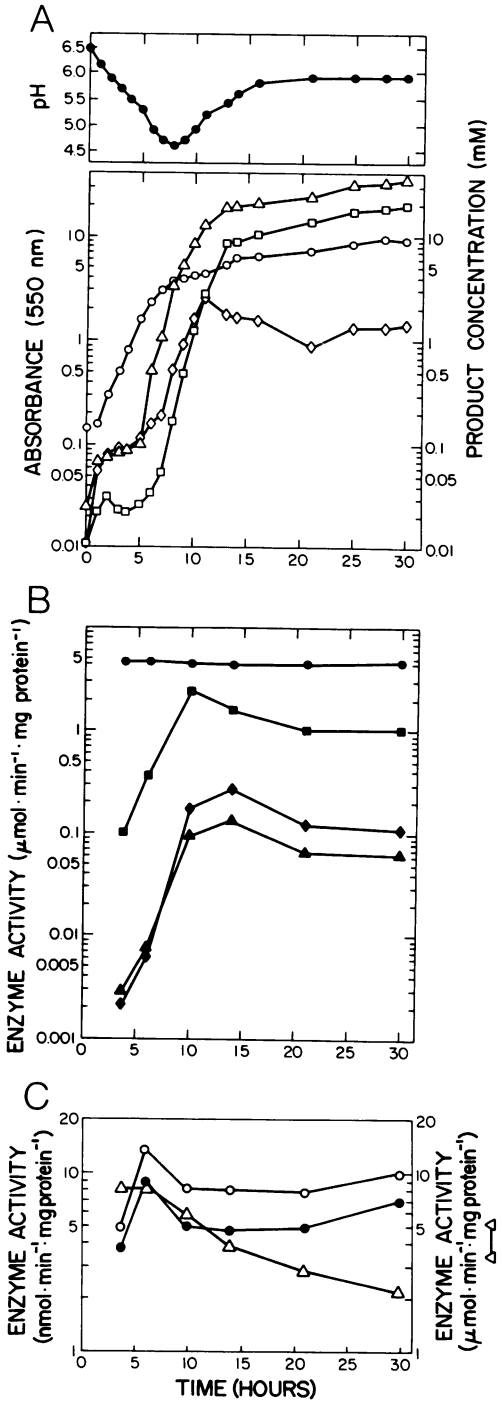

FIG. 3. Growth, culture pH, solvent production, and specific activity of solvent-forming enzymes of *C. beijerinckii* NRRL B593 in TYS medium. (A) A_{550} (○), acetone (◇), butanol (△), isopropanol (□), and pH (●). (B) Acetoacetate decarboxylase (■), butanol dehydrogenase (▲), isopropanol dehydrogenase (◆), and glucose-6-phosphate isomerase (●). (C) Acetoacetate-forming (acetoacetyl-CoA–utilizing) enzymes in the presence of potassium acetate (○) or potassium butyrate (●), and thiolase (△).

production about 2 h earlier (in relation to growth) than the previously assigned onset time (12). The culture pH at the onset of solvent production was 5.3 for both strains (Fig. 2A, 3A, and 4A), which is significantly higher than that usually

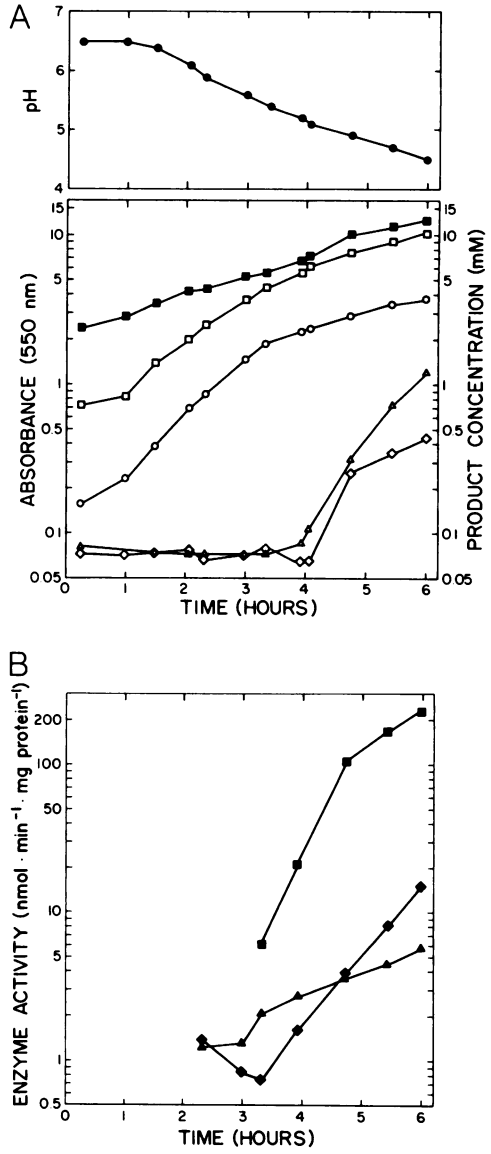

FIG. 4. Growth, culture pH, acid and solvent production, and specific activity of solvent-forming enzymes during the early phase of growth of *C. beijerinckii* NRRL B592. (A) A_{550} (○), acetate (■), butyrate (□), acetone (◇), butanol (△), and pH (●). (B) Acetoacetate decarboxylase (■), butyraldehyde dehydrogenase (◆), and butanol dehydrogenase (▲).

considered necessary for rapid solvent production (for a review, see reference 19). The total acid (dissociated plus undissociated acetate and butyrate) concentration in cultures at the onset of solvent production was 12 mM (6.5 mM acetate and 5.5 mM butyrate) for strain B592 (Fig. 4A), which is lower than the previously reported value (about 20 mM) based on a later onset time (12).

It may be expected that the level of at least some of these solvent-forming enzymes would increase before solvent production began, and this was indeed the case with both strains (Fig. 2 to 4). For strain NRRL B592, increases in these enzyme activities started at least 40 min earlier than the apparent onset of solvent production (Fig. 4). However, activities of all solvent-forming enzymes did not rise simultaneously. The extent and the pattern of increases in these enzyme activities were also different.

TABLE 1. Increase of specific activities of solvent-forming enzymes between the solvent-producing stage and the acid-producing stage in *C. beijerinckii* NRRL B592 and NRRL B593

Enzyme	Increase (fold)	
	Strain NRRL B592	Strain NRRL B593
Acetoacetate-forming enzyme(s)	2	3
Acetoacetate decarboxylase	165	24
Butyraldehyde dehydrogenase	25	ND[a]
Butanol dehydrogenase	5	46[b]
Isopropanol dehydrogenase	ND[a]	126

[a] ND, Not detectable. Isopropanol dehydrogenase is absent in strain NRRL B592 (17).

[b] It appears that a low level of a NAD(P)H-linked butanol-ethanol dehydrogenase activity (17) was present in acid-producing cells, and this activity did not increase significantly during the solventogenic switch. Thus, it could cause an apparent difference in the increase in butanol- and isopropanol-linked activities.

For the acetone-producing pathway of *C. beijerinckii* NRRL B592, the enzyme(s) which catalyzes acetoacetate formation from acetoacetyl-CoA was present in acid-producing (exponentially growing) cells (Fig. 2A and C). The specific activity of this enzyme(s) increased only twofold (Fig. 2C; Table 1), and the time of rapid increase paralleled the active solvent-producing period. The activity then decreased to below the level present in acid-producing cells (Fig. 2C). Acetoacetate decarboxylase was also present in acid-producing cells (Fig. 2A and B), and the activity started to increase when the activity of acetoacetate-forming enzyme(s) began to increase (Fig. 2B and C). The specific activity of acetoacetate decarboxylase increased 124-fold during the active solvent-producing period. When more frequent samples were taken in a separate experiment (Fig. 4B), acetoacetate decarboxylase activity was not detected in the first two samples and the activity was rising as soon as it was detected. Acetoacetate decarboxylase activity was assayed by manometry with a detection limit of 1.4 nmol/min per mg of protein. Therefore, it increased at least 165-fold during the 6-h experimental period (Fig. 4A and B; Table 1). (The manometric assay could not reliably measure a rate of gas production below 10 μl/30 min. With the protein concentration of cell extracts at about 30 mg/ml, the limit of the assay was 1.4 nmol/min per mg of protein.)

For the butanol-producing pathway of *C. beijerinckii* NRRL B592, butyraldehyde dehydrogenase and butanol dehydrogenase were present in acid-producing cells (Fig. 2A and B and 4A and B). The specific activity of butyraldehyde dehydrogenase decreased (Fig. 2B and 4B) before it started to increase about 40 min before the onset of butanol production (Fig. 4B). The specific activity of butanol dehydrogenase, however, started to increase near the mid-exponential phase of growth (Fig. 2A and B and 4A and B), at least 1 h earlier than the onset of butanol formation. The degree of increase for butyraldehyde dehydrogenase activity (25-fold) was much higher than that for butanol dehydrogenase activity (5-fold) (Table 1).

For *C. beijerinckii* NRRL B593, a fast rise in activities of solvent-forming enzymes occurred near the mid-exponential phase of growth (Fig. 3A to C). The acetoacetate-forming activity increased and then decreased to a relatively stable level, which is different from the mode found in strain NRRL B592. Thus, all solvent-forming enzyme activities, except the acetoacetate-forming activity in strain NRRL B593, decreased two- to fourfold between 15 and 30 h when solvent production slowed down. The pattern of changes for isopropanol dehydrogenase activity (which was present only in strain NRRL B593, the strain producing isopropanol) was similar to that for the butanol dehydrogenase activity of this organism, which might be expected because the two activities cannot be separated during purification (17). The increase in butanol dehydrogenase and isopropanol dehydrogenase activities paralleled each other except for the first two samples (Fig. 3B), which caused an apparent difference in the increase in these two activities (Table 1). The difference perhaps resulted from the presence of a minor, NAD(P)H-linked butanol-ethanol dehydrogenase in strain NRRL B593 (17), whose activity might not increase, whereas the NADPH-linked butanol-isopropanol-ethanol dehydrogenase activity did.

Thiolase activity was also measured in this study, because it might interfere with the assay for acetoacetate formation from acetoacetyl-CoA. Thiolase activity was high in both strains, and the level changed little in strain NRRL B592 during growth (Fig. 2C). However, it decreased with time in strain NRRL B593 (Fig. 3C). The distinct patterns of the activity levels of thiolase and the acetoacetate-forming enzyme(s) indicate that the latter pattern was not complicated by thiolase activity. The activity of a glycolytic enzyme, glucose-6-phosphate isomerase, was measured for comparison, and this activity remained relatively constant in both strains throughout the entire experimental period (Fig. 2B and 3B). Thus, the observed activity changes in solvent-forming enzymes were not caused by cellular changes that might affect the extraction and assay of enzymes.

The culture pH and acid concentrations were examined with respect to the earlier point of observed activity increase of solvent-forming enzymes in *C. beijerinckii* NRRL B592. This point for butanol dehydrogenase was at pH 5.7, and the acetate and butyrate (dissociated and undissociated) concentrations were 5.2 and 3.6 mM, respectively (Fig. 4A and B). The point for acetoacetate-forming enzyme(s), acetoacetate decarboxylase, and butyraldehyde dehydrogenase was at pH 5.3 (Fig. 2A to C). For *C. beijerinckii* NRRL B593, a fast rise in solvent-forming enzyme activities occurred at pH 5.5 (Fig. 3A to C). The previously recognized low pH (<5) for rapid solvent production is apparently not required for the onset of expression of solvent-forming enzymes or solvent production in *C. beijerinckii*.

DISCUSSION

All of the enzymes required for acetone-isopropanol and butanol synthesis (Fig. 1) have been detected in *C. beijerinckii* NRRL B592 and NRRL B593, except that butyraldehyde dehydrogenase activity was not detected in strain NRRL B593 and that isopropanol dehydrogenase activity was absent in strain NRRL B592. The activity of butyraldehyde dehydrogenase in strain NRRL B592 was NAD(H) specific and was measured by the following two assay systems: glycylglycine buffer (50 mM [pH 8 or 9]) containing 1 mM dithiothreitol, 3 mM NAD^+, 0.5 mM CoA, and 11 mM butyraldehyde, and Tris chloride buffer (50 mM [pH 7.5 or 8]), containing 3 mM NAD^+, 0.5 mM CoA, 100 mM KCl, and 11 mM butyraldehyde. When assayed in Tris chloride buffer the activity of butyraldehyde dehydrogenase was detected only when KCl was added. Butyraldehyde dehydrogenase activity in strain NRRL B593 was not detected by either procedure. Durre et al. (10) reported that the butyral-

dehyde dehydrogenase activity of *C. acetobutylicum* was best measured in the low-speed extract and with the physiological substrate. It remains to be shown whether this is also true with the *C. beijerinckii* enzyme. Since butanol dehydrogenase of *C. beijerinckii* NRRL B592 and NRRL B593 differ significantly (17), it may be speculated that their sequential metabolic enzyme (23, 24), butyraldehyde dehydrogenase, also differs significantly, so that the two dehydrogenases can interact closely to permit an efficient transfer of the slowly soluble metabolic intermediate, butyraldehyde, between the two enzymes.

The first step in the acetone-isopropanol–producing pathway is the formation of acetoacetate from acetoacetyl-CoA. We have considered several possible reactions for this step:

(i) CoA transfer (CoA transferase)

acetoacetyl-CoA + acetate or butyrate → acetoacetate + acetyl-CoA or butyryl-CoA

(ii) simple hydrolysis (acetoacetyl-CoA hydrolase)

acetoacetyl-CoA + H_2O → acetoacetate + CoA

(iii) via 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA synthase and HMG-CoA lyase)

synthase:

acetoacetyl-CoA + acetyl-CoA → HMG-CoA + CoA

lyase:

HMG-CoA → acetoacetate + acetyl-CoA

net reaction: acetoacetyl-CoA → acetoacetate + CoA

We did not detect any HMG-CoA synthase activity in extracts of solvent-producing cells by using a spectrophotometric assay (21), which suggests that HMG-CoA is not involved in this reaction (W.-L. Fan and J.-S. Chen, unpublished data). An acetoacetyl-CoA:acetate/butyrate CoA transferase activity has been demonstrated in cell extracts of *C. acetobutylicum* (2, 16) and *C. beijerinckii* (Hiu et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1985). More recently, we obtained evidence indicating that two activities, i.e., an acetoacetyl-CoA:acetate/butyrate CoA transferase activity and an acetoacetyl-CoA hydrolase activity, were present in *C. beijerinckii*, and the activity of acetoacetyl-CoA hydrolase was enhanced by potassium, rubidium, or ammonium ions (Yan and Chen, Fed. Proc., 1987; Yan and Chen, unpublished data). The acetoacetate-forming activity measured in cell extracts probably consists of both acetoacetyl-CoA:acetate/butyrate CoA transferase and acetoacetyl-CoA hydrolase activities because potassium, acetate, and butyrate are expected to be present in cell extracts. With thiolase also present in cell extracts, it was not practical to differentiate the two activities in this study, and the measured acetoacetate-forming activity was an overestimation, because thiolase would react with acetoacetyl-CoA and CoA (acetoacetyl CoA + CoA → 2 acetyl-CoA) when CoA was released by acetoacetyl-CoA hydrolase. The relative contribution of the two acetoacetate-forming activities to solvent production has not been determined, but our study of partially purified fractions showed that the relative levels of the hydrolase and the CoA transferase activities could be within a twofold range (R.-T. Yan, D. K. Thompson, and J.-S. Chen, unpublished data). We are now purifying the two enzymes further to determine their molecular properties.

It has been reported that acetoacetate decarboxylase in *C. acetobutylicum* can be induced by linear acids from C_1 to C_4 (at external concentrations above 5 mM and at pH 4.8), with acetoacetic acid being the most effective (3). However, it is not clear whether acetoacetic acid at pertinent intracellular concentrations is as effective. Our results show that (i) the formation of acetone follows the appearance of (or a large increase in) acetoacetate decarboxylase activity in *C. beijerinckii*, (ii) active acetone production paralleled a sustained increase in acetoacetate decarboxylase activity, and (iii) the activity increase in the acetoacetate-forming enzyme(s) was transient. These findings seem to suggest that the start of acetone production is controlled by the expression of acetoacetate decarboxylase activity and that the latter may require an increase in the activity of acetoacetate-forming enzyme(s). Thus, an increased expression of the acetoacetate-forming enzyme(s) is perhaps an early event of the solventogenic switch (for the acetone branch).

Butyraldehyde dehydrogenase and butanol dehydrogenase are enzymes specific for the butanol-forming pathway. With *C. acetobutylicum*, butyraldehyde dehydrogenase was not detectable in acid-producing cells (2, 10, 22), but low levels of butanol dehydrogenase were detected in acid-producing cells (10). Both butyraldehyde dehydrogenase and butanol dehydrogenase were detected in acid-producing *C. beijerinckii* cells. A low level of butanol dehydrogenase (4% of the highest activity) was detected in exponentially growing cells in CMC cultures of *C. beijerinckii* NRRL B592 two generations after outgrowth from spores (data not shown). It is interesting that in *C. beijerinckii* NRRL B592, the point at which the specific activity of butanol dehydrogenase began to increase was earlier than that of butyraldehyde dehydrogenase, although butanol dehydrogenase is the last step for butanol formation. It appears that in *C. beijerinckii* NRRL B592, butanol production occurs after the level of butyraldehyde dehydrogenase has increased, and the mode of regulation for butyraldehyde dehydrogenase and butanol dehydrogenase was different. In *C. acetobutylicum* DSM 1732, a difference in the pattern of activity changes for the two dehydrogenases was reported (10), but the scatter of the measured enzyme activities made the assignment of the onset of the activity increases difficult.

In summary, this study has shown that activities of all solvent-forming enzymes did not rise simultaneously in *C. beijerinckii*. The degree of increase in these enzyme activities also varied, with the acetoacetate-forming enzyme(s) (2- to 3-fold in both strains) showing the least and acetoacetate decarboxylase (≥165-fold in strain NRRL B592) and alcohol dehydrogenase (up to 126-fold in strain NRRL B593) showing the greatest increases (Table 1). Although the increase in acetoacetate-forming enzyme activity was not large, it could represent an early event of the solventogenic switch, and its expression might affect the expression of acetoacetate decarboxylase. These findings will be useful in the selection of solvent-forming genes to be studied in an effort to characterize promoters specific for solvent fermentation. Since the onset of expression of solvent-forming enzymes and the onset of solvent production are much earlier (in relation to growth) than the previously recognized time, investigations of extra- and intracellular conditions as possible triggering signals for the metabolic transition can now be carried out with cultures or cells at a more pertinent stage of growth. It may also be worthwhile to determine whether a differentiation can be

made between conditions that are most effective for triggering the onset of solvent production (enzyme expression) and conditions that are optimal for the ensuing solvent production.

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